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Haplotype and Phylogenetic Analyses Suggest That One European-Specific mtDNA Background Plays a Role in the Expression of Leber Hereditary Optic Neuropathy by Increasing the Penetrance of the Primary Mutations 11778 and 14484

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Summary

mtDNAs from 37 Italian subjects affected by Leber hereditary optic neuropathy (LHON) (28 were 11778 positive, 7 were 3460 positive, and 2 were 14484 positive) and from 99 Italian controls were screened for most of the mutations that currently are associated with LHON. High-resolution restriction-endonuclease analysis also was performed on all subjects, in order to define the phylogenetic relationships between the mtDNA haplotypes and the LHON mutations observed in patients and in controls. This analysis shows that the putative secondary/intermediate LHON mutations 4216, 4917, 13708, 15257, and 15812 are ancient polymorphisms, are associated in specific combinations, and define two common Caucasoid-specific haplotype groupings (haplogroups J and T). On the contrary, the same analysis shows that the primary mutations 11778, 3460, and 14484 are recent and are due to multiple mutational events. However, phylogenetic analysis also reveals a different evolutionary pattern for the three primary mutations. The 3460 mutations are distributed randomly along the phylogenetic trees, without any preferential association with the nine haplogroups (H, I, J, K, T, U, V, W, and X) that characterize European populations, whereas the 11778 and 14484 mutations show a strong preferential association with haplogroup J. This finding suggests that one ancient combination of haplogroup J–specific mutations increases both the penetrance of the two primary mutations 11778 and 14484 and the risk of disease expression.

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Introduction

Leber hereditary optic neuropathy (LHON) is a maternally transmitted disease in which the primary clinical manifestation is acute or subacute bilateral loss of central vision, leading to central scotoma and blindness. Since 1988, when the first LHON mutation in human mtDNA was discovered, a growing number of mtDNA missense mutations have been associated with the disease, and a total of 17 LHON mtDNA mutations now are reported in the literature (Wallace et al. 1995). On the basis of numerous genetic, clinical, and biochemical criteria, mutations at three nucleotide positions (nps), 11778 (Wallace et al. 1988), 3460 (Howell et al. 1991*a*; Huoponen et al. 1991), and 14484 (Johns et al. 1992, 1993*a*; Mackey and Howell 1992), are considered as high-risk and primary LHON mutations (Newman 1993; Brown and Wallace 1994). Overall, these three mutations encompass a large majority of LHON families in all human populations (Ishikawa et al. 1995; Savontaus 1995). The pathological significance of the other LHON mutations is much less clear, and it remains to be determined whether they cause the disease, contribute to the pathology, or are nonpathogenic mtDNA polymorphisms (Riordan-Eva and Harding 1995). The 13730 (Howell et al. 1993*a*) and 9438 and 9804 (Johns and Neufeld 1993) mutations also may be primary mutations, but they have been reported in only one or a few LHON families, and their pathological significance requires confirmation. The 4160 mutation initially was associated with LHON in a LHON-like pedigree with neurological abnormalities (Howell et al. 1991*b*), but, more recently, it has been suggested that it may be responsible only for the neurological abnormalities (Howell 1994). The mutations 3394 (Brown et al. 1992*a*; Johns et al. 1992), 4136 (Howell et al. 1991*b*), 4216 (Johns and Berman 1991; Brown et al. 1992*a*), 4917 (Johns and Berman 1991), 5244 (Brown et al. 1992*a*), 7444 (Brown et al. 1992*b*), 9901 (Lamminen et al.

1995), 13708 (Johns and Berman 1991; Brown et al. 1992*a*), and 15257 and 15812 (Johns and Neufeld 1991; Brown et al. 1992*a*) generally are considered to be less severe LHON mutations (secondary/intermediate mutations). They are homoplasmic and are associated with each other and with primary mutations. They also are observed in control populations, although at a lower frequency than in LHON populations, and appear to be more common in Europeans or in subjects of European ancestry than in other populations (Ishikawa et al. 1995).

To obtain some additional information concerning the role played by the mtDNA mutations currently associated with LHON, we performed a molecular study based on three interrelated steps. First, we performed a screening for 15 mutations associated with LHON in ethnically matched patient and control populations (37 Italian patients and 99 Italian controls). Second, we performed high-resolution haplotype analyses of mtDNA from all patients and controls. Third, we established the phylogenetic relationships of the mutations associated with LHON. The resulting data suggest that one common European-specific mtDNA haplogroup plays a role in the expression of two of the three most common primary LHON mutations and that this most likely is due to one ancient combination of mutations, which characterizes this mtDNA background.

Subjects and Methods

Subjects

Of the 37 LHON subjects analyzed (L1–L37), 16 were collected in Milan, 12 in Rome, and 9 in Bologna, and all were diagnosed as LHON cases on the basis of a clinical examination and molecular screening for the three primary mutations 11778, 3460, and 14484 (28 were 11778 positive, 7 were 3460 positive, and 2 were 14484 positive). They include all subjects available at the time of the beginning of the study, and there was no preferential selection for any of the three classes of patients. Thus, the observed frequencies should reflect the relative proportion of the 11778, 3460, and 14484 mutations among Italian LHON cases. Nineteen of the 37 LHON cases (L2, L3, L5, L6, L8, L9, L13, L17, L18, L20, L21, L24, L27–L29, L31, L32, L35, and L37) were classified as having a positive family history. This group includes subjects with maternal relatives who had been diagnosed previously as LHON cases and subjects from previously undiagnosed pedigrees that, after clinical examination of additional family members, showed at least another affected relative. The families that, according to anamnestic reports from the proband or from other relatives, harbored other maternally related members with visual problems compatible with a LHON diagnosis, even though these subjects were not available for examination, also were included in this group. The remaining 18 cases were classified as singletons. Although careful evaluation of familial anamnestic data failed to detect affected relatives in the pedigrees, we cannot rule out definitively that they included some unrecognized familial cases, because genealogical records were not available (Mackey 1994). Interviews with the probands indicated that all subjects were of Italian ancestry (11 from northern Italy, 12 from central Italy, 6 from southern Italy, 5 from Sicily, and 3 from Sardinia) and apparently were unrelated. DNA and blood samples from these LHON cases had been accumulated, during the course of several years, without any form of selection. The 99 unrelated Italian controls used in this study included a sample of 51 continental Italians and 48 Sardinians. Total DNA was extracted from buffy coats by use of standard methods.

Haplotype Analysis and LHON Mutation Screening

The entire mtDNA of each sample was amplified in nine overlapping fragments by use of PCR and primer pairs 1–9 listed in table 1. Each of these nine PCR segments was digested with 14 restriction endonucleases (*Alu*I, *Ava*II, *Bam*HI, *Dde*I, *Hae*II, *Hae*III, *Hha*I, *Hin*cII, *Hin*fI, *Hpa*I, *Msp*I, *Mbo*I, *Rsa*I, and *Taq*I). This restriction analysis screens ~20% of mtDNA nucleotides and defines the mtDNA haplotype (Torroni et al. 1993*a*, 1993*b*, 1994*a*, 1994*b*, 1994*c*, 1994*d*). Among the 15 LHON mutations studied, four mutations—ND1-3394C, ND2-5244A, COIII-9438A, and Cyt b-15812A—change a *Hae*III, *Msp*I, *Hae*III, and *Rsa*I restriction site, respectively, and were tested for during the mtDNA haplotype analysis. For the remaining 11 mutations, the screening was performed either by generation of additional PCR fragments or by use of the PCR fragments already synthesized for the haplotype analysis. The ND1-3460A, ND4-11778A, and ND6-14484C mutations were detected by the screening of the PCR fragments generated with primer pairs 10, 11, and 12 and the enzymes *Bsa*HI, *Mae*III, and *Bst*OI, respectively. For the 14484-mutation screening, a reverse mismatched primer was created (table 1). The ND1-4136G, ND1-4216C, and ND2-4917G mutations were detected by the screening of the PCR fragment generated with primer pair 3 and the enzymes *Nla*III, *Nla*III, and *Bfa*I, respectively. Primer pair 5 and the enzyme *Xba*I were used for the COI-7444A mutation. Primer pair 6 and the enzyme *Mae*III were used for the COIII-9804A mutation. The putative LHON mutation ND4L-10663C (Brown et al. 1995) was analyzed with primer pair 7 and the enzyme *Spe*I. The ND5-13708A mutation was tested with primer pair 8 and the enzyme *Bst*OI. Finally, primer pair 9 and the enzyme *Acc*I were used for the Cyt b-15257A mutation. Additional polymorphic sites detected during the screening for the LHON-associated mutations were included in the haplotypes (see Appendix). Restriction fragments were resolved by electropho-

Table 1

PCR Primers Used for Amplification of the Entire mtDNA of Italian LHON Patients and Controls and of the mtDNA Regions Encompassing Specific Mutations Associated with LHON

Primer Pair	Coordinates	Annealing Temperature[a] (°C)	Segment Size (bp)
1	16453–16472, 1696–1677	60	1,813
2	1615–1643, 4102–4084	53	2,488
3	3816–3835, 5193–5174	50	1,378
4	5151–5170, 6263–6247	50	1,113
5	6149–6168, 8478–8455	53	2,330
6	8416–8436, 10107–10088	55	1,692
7	9911–9932, 12338–12309[b]	55	2,428
8	12190–12209, 14385–14366	55	2,196
9	14260–14279, 16543–16526	51	2,284
10	3108–3127, 3717–3701	51	609
11	11141–11158, 11868–11851	51	728
12	14191–14210, 14509–14485[c]	60	319

NOTE.—Primers are numbered according to the study by Anderson et al. (1981); the 5′→3′ coordinates before the comma correspond to the forward primers and those after the comma correspond to the reverse primers. Oligonucleotide pairs 1–9 were used for the haplotype analysis and for the secreening of all mutations associated with LHON, except for the 3460, 11778, and 14484 mutations. These three mutations were screened by use of oligonucleotide pairs 10, 11, and 12, respectively.

[a] The temperature used was the lowest for the primer pair.

[b] The reverse primer of this pair of oligonucleotides was altered in order to screen for an A→G transition at np 12308 in the $tRNA^{Leu}$ gene. Screening for the 12308 mutation involved creation of a reverse primer with the 3′ end adjacent to np 12308 and with a mismatched guanine at np 12312. In the presence of this mismatch, the 12308G mutation determines the creation of a *Hin*fI site.

[c] The reverse primer of this pair of oligonucleotides was altered in order to screen for a T→C transition at np 14484 in the ND6 gene. Screening for the 14484 mutation involved creation of a reverse primer with the 3′ end adjacent to np 14484 and with two mismatched cytosines at nps 14487 and 14488. In the presence of this mismatch, the 14484C mutation determines the creation of a *Bst*OI site.

resis in NuSieve plus SeaKem agarose gels (FMC BioProducts) and were visualized by UV-induced fluorescence.

Phylogenetic Analysis and Sequence-Divergence Estimations

The phylogenetic relationships between haplotypes were inferred by use of parsimony analysis. The dendrograms were rooted by use of a Senegalese mtDNA haplotype (called the "African outgroup"; Torroni et al. 1993*a*). Maximum-parsimony (MP) trees were generated by random addition of sequences, by use of the Tree Bisection and Reconnection (TBR) algorithm (Phylogenetic Analysis Using Parsimony [PAUP], version 3.1.1; Swofford 1993). Because of the large number of terminal taxa, thousands of MP trees could be obtained. We terminated our searches at 3,000 trees after 444 replications and saved ≤10 MP trees for each replication. Although shorter trees could exist, none were observed in our analyses. A strict-consensus tree of the 3,000 MP trees generated by the TBR algorithm was constructed. We performed parsimony analyses by both including and excluding secondary/intermediate LHON mutations. However, since their inclusion did not affect the location and relationship of the haplotypes, only the phylogenetic trees derived by the inclusion of these mutations are shown. Primary mutations were not used for tree construction and were simply superimposed on final dendrograms.

Intrahaplogroup sequence-divergence estimates were calculated by use of the maximum-likelihood procedure of Nei and Tajima (1983). When the divergence times of the haplogroups were calculated, a 2.2%–2.9%/million-years rate of mtDNA evolution was used (Torroni et al. 1994*d*).

Results

Screening for Mutations Associated with LHON in Italian Patients and Controls

The restriction-analysis screening of LHON subjects has shown that the three primary mutations 11778, 3460, and 14484 were not in association with each other and were absent among the controls. Among probands, heteroplasmy was observed in 8 (28.6%; cases L2, L8, L11, L12, L17, L21, L23, and L30) of the 28 11778 cases, in 3 (42.9%; cases L4, L14, and L16) of the 7 3460 cases, and in 1 (50%; case L34) of the 2 14484 cases. These figures are higher than those observed in other LHON populations, particularly for the 3460 mutation, and became even higher (32.1% and 85.7% of the 11778 and 3460 cases, respectively) when heteroplasmy was assayed in family members. This is because some members of the family of proband L13 (homoplasmic for the 11778 mutation) were heteroplasmic, and heteroplasmy of the 3460 mutation was observed among members of the families of the homoplasmic singleton probands L3, L15, and L36. However, similar frequencies have been reported by Oostra et al. (1994), who observed heteroplasmy in five (62.5%) of eight 3460-positive pedigrees.

The screening for secondary/intermediate LHON mutations showed different frequency patterns among the three classes of patients (11778 positive, 3460 positive, and 14484 positive) and controls (table 2). The 4917 mutation had high and virtually identical frequencies in patients (8.1%) and in controls (9.1%). The 4216 and 13708 mutations showed an extremely high frequency in patients who were 11778 positive (50.0% and 39.3%, respectively) and in patients who were 14484 positive (100.0% for both mutations), but they also were very common among controls (16.2% and 8.1%, respectively). Similarly, the 15257 and 15812 mutations showed high frequencies in the 11778-positive patients

Table 2

Frequency of Secondary/Intermediate LHON Mutations in Italian Patients and Controls

Mutation[a]	Classification[b]	No. (%) of LHON Patients, by Primary Mutation				No. (%) of Controls		
		11778 [N = 28]	3460 [N = 7]	14484 [N = 2]	Total [N = 37]	Italians [N = 51]	Sardinians [N = 48]	Total [N = 99]
4136	Secondary	2 (7.1)	0	0	2 (5.4)	0	0	0
4216	Secondary	14 (50.0)	0	2 (100.0)	16 (43.2)	9 (17.6)	7 (14.6)	16 (16.2)
4917	Secondary	3 (10.7)	0	0	3 (8.1)	5 (9.8)	4 (8.3)	9 (9.1)
13708	Secondary	11 (39.3)	1 (14.3)	2 (100.0)	14 (37.8)	4 (7.8)	4 (8.3)	8 (8.1)
15257	Intermediate	4 (14.3)	0	0	4 (10.8)	3 (5.9)	2 (4.2)	5 (5.1)
15812	Secondary	3 (10.7)	0	0	3 (8.1)	1 (2.0)	2 (4.2)	3 (3.0)

[a] The 3394, 5244, 7444, 9438, 9804, and 10663 mutations were not observed in either patients or controls.
[b] According to the study by Brown et al. (1995).

(14.3% and 10.7%, respectively) but also were commonly observed in controls (5.1% and 3.0%, respectively). The 4136 secondary mutation was observed only in two 11778-positive patients (7.1%). All these secondary/intermediate LHON mutations were homoplasmic. Among the 15 previously identified LHON mutations assayed in this screening, 6 mutations were not observed in either patients or controls; these are the 3394, 5244, 7444, 9438, 9804, and 10663 mutations.

Table 3 shows the combinations of secondary/intermediate LHON mutations observed in the three groups of patients and in the controls and confirms that LHON mutations are not distributed randomly among haplotypes (Brown et al. 1992*a*). The 15812 mutation always was associated with the 15257 mutation, which, in turn, always was associated with the 13708 mutation. Moreover, the 13708 mutation always was associated with the 4216 mutation. On the contrary, the 4216 mutation also was observed in a second, alternative association, which is defined by the presence of the 4917 mutation. The combinations 4216 + 13708, 4216 + 13708 + 15257, and 4216 + 13708 + 15257 + 15182 (see shaded box in table 3) are more frequent among the 11778- and the 14484-positive patients than among the controls, thus explaining the increased frequency of each of these mutations among LHON subjects. Three exceptions to this association phenomenon were observed. The 13708 and 15257 mutations were associated independently with the 4216 + 4917 combination, each in one control subject, whereas the 13708 mutation also was observed in one 3460-positive subject lacking other secondary mutations.

Haplotype and Phylogenetic Analysis of mtDNA from LHON Patients and Controls

High-resolution restriction analysis revealed 30 haplotypes among the 37 LHON patients (L1–L37) and 64 haplotypes (1–64) among the 99 Italian controls. Two

Table 3

Combinations of Secondary/Intermediate LHON Mutations in Italian Patients and Controls

Combination Status[a]						No. (%) of LHON Patients, by Primary Mutation				No. (%) of Controls		
15257	13708	4216	4917	4136	15812	11778 [N = 28]	3460 [N = 7]	14484 [N = 2]	Total [N = 37]	Italians [N = 51]	Sardinians [N = 48]	Total [N = 99]
−	−	−	−	−	−	14 (50.0)	6 (85.7)	0	20 (54.1)	42 (82.4)	41 (85.4)	83 (83.8)
−	+	−	−	−	−	0	1 (14.3)	0	1 (2.7)	0	0	0
−	+	+	−	−	−	7 (25.0)	0	2 (100.0)	9 (24.3)	2 (3.9)	1 (2.1)	3 (3.0)
+	+	+	−	−	−	1 (3.6)	0	0	1 (2.7)	1 (2.0)	0	1 (1.0)
+	+	+	−	−	+	3 (10.7)	0	0	3 (8.1)	1 (2.0)	2 (4.2)	3 (3.0)
−	−	+	+	−	−	1 (3.6)	0	0	1 (2.7)	4 (7.8)	3 (6.3)	7 (7.1)
−	−	+	+	+	−	2 (7.1)	0	0	2 (5.4)	0	0	0
+	−	+	+	−	−	0	0	0	0	1 (2.0)	0	1 (1.0)
−	+	+	+	−	−	0	0	0	0	0	1 (2.1)	1 (1.0)

Note.—The shaded area indicates combinations that are much more frequent in patients than in controls.
[a] A plus sign (+) indicates presence of the secondary/intermediate mutation; a minus sign (−) indicates absence of the secondary/intermediate mutation.

of the haplotypes observed in the patients were differentiated further by the distinguishing presence of the primary LHON mutations 11778, 3460, and 14484, thus bringing the total number of patient haplotypes to 32. All together these haplotypes were defined by 107 polymorphic restriction sites (see Appendix), including LHON mutations 11778, 3460, and 14484, and by the 9-bp COII/tRNA^{Lys} intergenic deletion (Torroni et al. 1995), the 9-bp triplication in the same region (Passarino et al. 1993), and the 12S rRNA gene insertion of five cytosines in the region between nps 956 and 965, which was described only in one Alzheimer-plus-Parkinson–disease patient (Shoffner et al. 1993). Whereas both variations in the intergenic region between COII and tRNA^{Lys} were observed in the controls, the 12S rRNA insertion was observed in one LHON subject (L5).

Parsimony analysis revealed that, similar to other European populations (Torroni et al. 1994*b*, 1996*b*), a large majority of Italian mtDNA can be aggregated into nine major haplotype groups (haplogroups H, I, J, K, T, U, V, W, and X) (fig. 1). The overall validity and robustness of the haplogroup aggregation is confirmed by the strict-consensus tree, in which all haplogroups, except haplogroups K and X, are retained (see inset in fig. 1). Previous analyses also have indicated that haplogroups H, I, K, J, T, V, and W are Caucasoid specific, whereas haplogroups U and X are shared between Caucasoid and non-Caucasoid populations (Torroni et al. 1994*b*, 1996*b*). Among the Italian haplotypes, one haplotype (64) belonging to the African-specific haplogroup L (Chen et al. 1995) and one haplotype (63) belonging to the Asian-specific haplogroup M (Torroni et al. 1994*c*; Wallace 1995) (fig. 1) were observed. Only six (4.4%) of the mtDNA samples observed in Italy lacked any informative restriction-site change and did not cluster in any of the known haplogroups (labeled "Others" in fig. 1).

The Phylogenetic Distribution of Mutations Associated with LHON

Secondary/intermediate mutations.—Parsimony analysis shows that the 4216 + 4917 and the 4216 + 13708 combinations are monophyletic and, together with several other polymorphic sites, define all mtDNA belonging to haplogroups T and J, respectively, which are two of the European-specific haplogroups (fig. 1). Such a phylogenetic separation of haplogroups T and J is supported by the strict-consensus tree (fig. 1) and by previous studies (Brown et al. 1992*a*, 1995; Torroni et al. 1994*b*, 1996*b*), thus indicating that the 4216 mutation has occurred twice in Europeans. However, other phylogenetic analyses of mtDNA sequences have suggested that haplogroups T and J could be two ancient subclusters of the same major mtDNA lineage and that the 4216 mutation would have occurred once (Howell et al. 1995; Richards et al. 1996). Two additional occurrences of the 13708 mutation also were observed. One occurrence was in the mtDNA from a control belonging to haplogroup T, and one was in the mtDNA of a 3460-positive patient (L19) belonging to haplogroup X (fig. 1).

Analysis of the associations of LHON mutations (table 3) showed that all but one of the 15257 mutations were found in association with the 4216 + 13708 combination and that all 15812 mutations were associated with the 4216 + 13708 + 15257 combination. Figure 1 confirms that the 4216 + 13708 + 15257 combination defines a monophyletic cluster within haplogroup J and that the 15812 mutation characterizes a subset of this cluster. Thus, the mutational event that generated the 15812 mutation is more recent than that which gave rise to the 15257 mutation, which, in turn, occurred after the mutational events that originated the 4216 + 13708 combination. Figure 1 also illustrates that the 15257 mutation not associated with the 4216 + 13708 combination was due to an independent mutational event on a haplogroup T mtDNA.

The 4136 mutation was observed only in two LHON subjects (L10 and L11) harboring the same haplogroup T haplotype (fig. 1). Both of them were characterized by the 11778 mutation, and, in subject L11, the 11778 mutation was heteroplasmic. The families were from different towns in southern Italy, and interviews with members of the two families were not able to establish any maternal link between the families. However, since the 4136 mutation is rare, its presence on the same haplotype suggests that families L10 and L11 actually are related and share the mutation by descent.

The 3460 primary mutation.—Parsimony analysis revealed that the seven mtDNA samples harboring the 3460 mutation are scattered widely on phylogenetic trees clustering within five different haplogroups (fig. 1). Two of the 3460-positive cases (L3 and L4) are members of haplogroup H, two (L14 and L36) belong to haplogroup U, and one each belongs to haplogroups V (L16), X (L19), and W (L15). This distribution immediately reveals that at least five of the seven 3460 mutations are due to independent mutational events. Because the two LHON mtDNA samples belonging to haplogroup U harbor different haplotypes, they also can be attributed to two independent mutational events, thus bringing the total number of recognizable mutational events to six. The random distribution of the 3460 mutation among different mtDNA backgrounds is confirmed by the lack of any significant difference between the frequency of each haplogroup in the general Italian population and that of the 3460 mutation in the same haplogroups (table 4). The recent origin of the 3460 mutations observed in Italian patients is confirmed by the high proportion (85.7%; see fig. 1) of 3460-positive heteroplasmic families, since heteroplasmy usually is considered an indicator of the recent occurrence of an mtDNA mutation.

The 11778 primary mutation.—Similar to the 3460

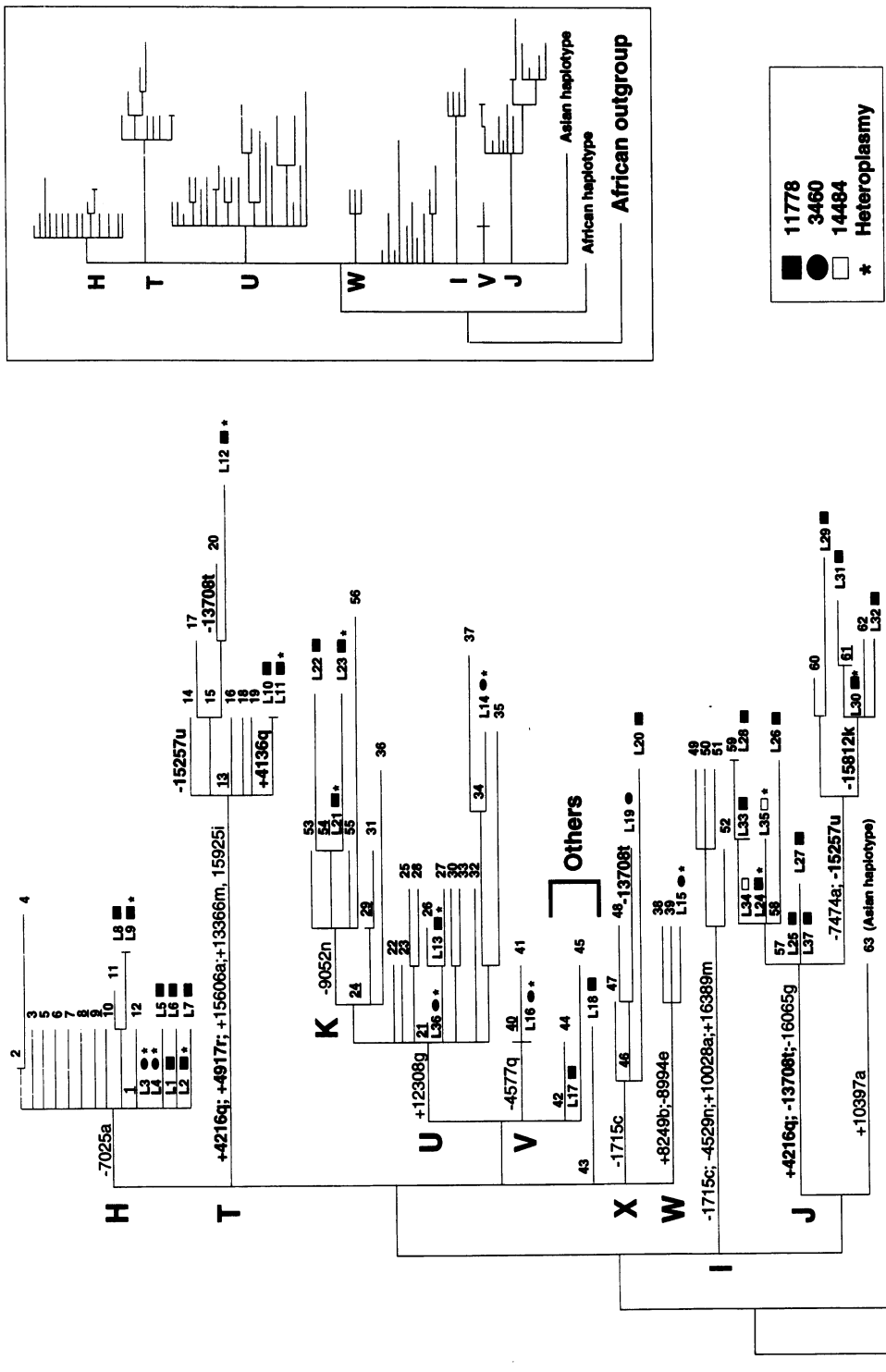

Figure 1 Parsimony tree of Italian mtDNA from LHON patients and from controls. This MP tree was obtained by PAUP analysis and includes the haplotypes observed in 37 LHON patients (L1–L37) and the 64 haplotypes (1–64) observed in 99 Italian controls (51 continental Italians and 48 Sardinians). The number(s) at the node or at the end of each branch indicates mtDNA haplotypes observed in individual LHON patients (L designation) and/or haplotypes observed in controls. The 19 LHON cases with a positive family history are L2, L3, L5, L6, L8, L9, L13, L17, L18, L20, L21, L24, L27–L29, L31, L32, L35, and L37. For the patients, all mtDNA haplotypes are shown, and the presence of either primary mutations or heteroplasmy is shown (see key). Underlined numbers indicate the 10 haplotypes observed in >1 individual among the 99 controls. The number of individuals for each of these haplotypes is indicated in parentheses as follows: 1 (20), 8 (2), 9 (2), 13 (2), 21 (2), 24 (4), 29 (2), 40 (4), 54 (5), and 61 (2). The tree was rooted by use of mtDNA from a Senegalese subject (Torroni et al. 1993*a*). The capital letters H, I, J, K, T, U, V, W, and X indicate nine haplogroups, and the numbers associated with the lowercase letters indicate either restriction sites defining specific haplogroups or the presence of secondary LHON-associated mutations (indicated by boldface). The restriction enzymes correspond to the following code: a = *AluI*; b = *AvaII*; c = *DdeI*; e = *HaeIII*; g = *HinfI*; k = *RsaI*; i= *MspI*; m = *BamHI*; n = *HaeII*; q = *NlaIII*; r = *BfaI*; t = *BstOI*; and u = *AccI*. Haplotypes that lack characteristic mutations and that do not form haplogroups are labeled "Others." The horizontal branch lengths are proportional to the number of mutational events that separate the haplotypes, with the exception of the 16517 *HaeIII* site. To this hypervariable site has been assigned a weight that is half that assigned to all other sites. This tree is 274 steps in length, has consistency and retention indices of .617 and .903, respectively, and is 1 of the 3,000 MP trees generated with the TBR branch-swapping algorithm. The strict-consensus tree of the 3,000 MP trees is illustrated in the inset and is 315 steps in length, with consistency and retention indices of .478 and .829, respectively. All haplogroups but haplogroups K and X are retained in the consensus tree.

Table 4

Haplogroup Distribution of mtDNA from Italian LHON Patients and Controls

Group and Primary-Mutation Subgroup	N^a	n^b	No. (%) of mtDNA Samples in Haplogroup									
			H	I	J	K	T	U	V	W	X	Others
LHON patients:												
11778	28	24	7 (25.0)	...	11 (39.3)	3 (10.7)	3 (10.7)	1 (3.6)	...	...	1 (3.6)	2 (7.1)
3460	7	6	2 (28.6)	...	...	...	...	2 (28.6)	1 (14.3)	1 (14.3)	1 (14.3)	...
14484	2	2	...	...	2 (100.0)	...	...	...	...	...	...	...
Total	37	32	9 (24.3)	...	13 (35.1)	3 (8.1)	3 (8.1)	3 (8.1)	1 (2.7)	1 (2.7)	2 (5.4)	2 (5.4)
Controls	99	64	33 (33.3)	4 (4.0)	7 (7.1)	8 (8.1)	9 (9.1)	22 (22.2)	5 (5.1)	2 (2.0)	3 (3.0)	6 (6.1)

NOTE.—The shaded areas encompass the data for the haplogroups that are differentially distributed among patients and controls.

[a] Number of individuals in each group of subjects.

[b] Number of different mtDNA haplotypes observed in each group of subjects.

mutation, the 11778 mutation was observed in mtDNA samples belonging to a wide range of haplogroups, including haplogroups H, J, K, T, U, and X, and to the group called "Others" (fig. 1 and table 4). Twenty-four different haplotypes were observed among the 28 mtDNA samples harboring this mutation, thus suggesting that the 11778 mutation has been generated by an extensive number of independent mutational events in our population sample. The multiple and recent origin of this primary mutation is supported by the high incidence (32.1%) of heteroplasmy in the 11778-positive families. These findings are in agreement with the expectations for a severe mutation. However, a detailed analysis of the haplogroup distribution of the 11778 mutation relative to the Italian control population identifies a feature that greatly distinguishes the 11778 and 3460 mutations. The distribution of the 11778 mutation in different mtDNA backgrounds is not random and shows a preferential association with haplogroup J, one of the European-specific haplogroups (table 4). Haplogroup J encompasses 39.3% of the 11778-positive mtDNA samples, whereas it represents only 7.1% of the Italian mtDNA samples. Thus, in this haplogroup, the incidence of the 11778 mutation is five to six times higher than would be expected if the 11778 mutation had occurred in all mtDNA backgrounds randomly, and this difference is highly significant ($P = .0001$) by the two-tailed Fisher's exact test. A subdivision of haplogroup J LHON cases into familial and sporadic cases indicates that the increased frequency of the 11778 mutation in this haplogroup is caused by an equal increase of both of these classes in haplogroup J mtDNAs (7 familial cases vs. 4 sporadic cases) relative to non–haplogroup J mtDNAs (10 familial cases vs. 7 sporadic cases). An opposite trend for the distribution of the 11778 mutation is observed for haplogroup U. This haplogroup encompasses 22.2% of the control mtDNA samples but only 3.6% of the 11778-positive mtDNA samples ($P = .0251$).

The 14484 primary mutation.—The two 14484-positive cases (L34 and L35) harbored two different haplotypes that both belonged to haplogroup J. In family L34, the proband and four unaffected maternal relatives were heteroplasmic, whereas the proband from family L35 and his two affected brothers were all homoplasmic for the 14484 mutation. These findings suggest that the 14484 mutations in the two families are due to independent mutational events in haplogroup J mtDNAs.

Discussion

Secondary/Intermediate LHON Mutations

The analysis of secondary/intermediate LHON mutations in the Italian population shows that the mutations are in specific monophyletic associations, characterize specific haplogroups (fig. 1), and commonly are observed in the control Italian population. These findings are compatible with those expected for low-risk/neutral polymorphisms (Torroni and Wallace 1994). However, the different frequency values observed, in patients and controls, for different secondary/intermediate LHON mutations indicate that these mutations can be subdivided into three classes.

The first class is represented by the 4917 mutation, which always is observed in the 4216 + 4917 combination. This combination shows virtually identical frequencies in patients (8.1%) and in controls (9.1%) and, together with several restriction-site changes, defines haplogroup T, one of the European-specific haplogroups (fig. 1). The 4917 mutation and the 4216 + 4917 combination are very ancient (table 5), implying transmission for 1,000–1,500 generations. The ancient origin of haplogroup T is supported by its wide distribution among Caucasoid populations, with a frequency of 4.4% in the Druze, 14.0% in the Adygei of southern Russia, 6.0% in the Basques, 6.1% in the Finns, and 21.6% in the Swedes (Torroni et al.1996*b*; A. Torroni, unpublished data). These features are not compatible

Table 5

Sequence Divergences and Ages of mtDNA Haplogroups J and T in Italy

Haplogroup (Mutation Combination)	No. of Haplotypes	No. of Subjects	Sequence Divergence (%)	Divergence Time[a] (Years)
J (4216 + 13708)	14	20	.102	35,000–46,000
J_2 (4216 + 13708 + 15257)	7	8	.066	23,000–30,000
$J_{2.2}$ (4216 + 13708 + 15257 + 15812)	5	6	.035	12,000–16,000
T (4216 + 4917)	10	12	.064	22,000–29,000

NOTE.—The 11778, 3460, and 14484 mutations were not included in the calculations of the intrahaplogroup sequence divergences.

[a] Calculated from intrahaplogroup sequence divergences, by use of the mtDNA evolution rate of 2.2%–2.9%/million years (Torroni et al. 1994*d*).

with hypotheses suggesting that either the 4917 mutation or the 4216 + 4917 combination might play a role in LHON expression.

The second class encompasses the 4136 mutation. We have observed this mutation only in two 11778-positive patients harboring the same haplogroup T haplotype. The 4136 mutation was described elsewhere in a subbranch of an Australian LHON pedigree, in association with the 4160 and 14484 mutations, and it was suggested that the 4136 mutation might function as an intragenic suppressor ameliorating the effects of the associated LHON mutations (Howell et al. 1991*b*). The Australian mtDNA lacked the 4216 and 4917 mutations, which characterize the two Italian LHON families, thus indicating that it did not belong to haplogroup T and that the Australian and Italian 4136 mutations are due to independent mutational events. To further assess the frequency of this mutation in human populations, we also analyzed 200 control subjects belonging to various Caucasoid groups, in addition to the Italian controls. None of the controls harbored the 4136 mutation, suggesting that it is rather uncommon. These findings neither support nor dismiss the possibility that the 4136 mutation is an ameliorating mutation, but we find it to be unusual that this mutation has been found only in LHON families. This feature suggests that the 4136 mutation has a role in causing LHON, rather than in protecting from LHON.

The third, and most numerous, class of secondary/intermediate LHON mutations encompasses the 4216 + 13708 combination and the 15257 and 15812 mutations. These mutations are all common in controls but show much higher frequencies in patients (table 2 and table 3). Phylogenetic analysis confirms that the 4216 + 13708 combination is ancient (table 5) and monophyletic (Brown et al. 1995; Howell et al. 1995), and it defines haplogroup J, one of the European-specific haplogroups (Torroni et al. 1994*b*). The origin of this haplogroup in an ancient Caucasoid population is supported by its distribution in modern populations. This haplogroup also has been observed in the Yemenite Jews (26.3%), the Druze (6.7%), the Adygei (4.0%), the Basques (2.0%), the Finns (14.3%), and the Swedes (2.7%) (Torroni et al. 1996*b*; A. Torroni, unpublished data).

Haplotype and phylogenetic analyses confirm that the intermediate LHON mutation 15257 also is monophyletic (Brown et al. 1992*a*), has occurred in an ancestral haplogroup J mtDNA (fig. 1), and now defines a specific subset of haplotypes termed "J_2" (fig. 2). Even if more recent than the 4216 + 13708 combination, this mutation still is ancient, having originated 23,000–30,000 years ago (table 5). In addition to the Italian control population, haplotypes belonging to J_2 have been described among the Druze (4.4%) and the Finns (2.0%) and have reached a particularly high frequency among the Yemenite Jews (17.9%) (Torroni et al. 1996*b*; A. Torroni, unpublished data). Finally, the 15812 mutation also is monophyletic and occurred on J_2 mtDNA, which already was characterized by the 4216 + 13708 + 15257 combination. This mutation originated 12,000–16,000 years ago (table 5) and defines a subset of J_2, which we have termed "$J_{2.2}$" (fig. 2). Population screening in Caucasoid populations suggests that $J_{2.2}$ has a much more limited geographic distribution than J or J_2, since, among all the Caucasoid populations listed previously, $J_{2.2}$ was observed only in continental Italians and in Sardinians.

The monophyletism, the age, and the high frequency, in Caucasoid populations, of this third class of mutations all are compatible with the evolutionary features of low-risk/neutral mutations. However, it remains to be explained why the secondary/intermediate LHON mutations that are haplogroup J specific are more frequent in LHON patients than in controls.

Primary LHON Mutations

Parsimony analysis indicates that the 11778, 3460, and 14484 mutations have appeared in a large number of different mtDNA haplotypes. In agreement with the

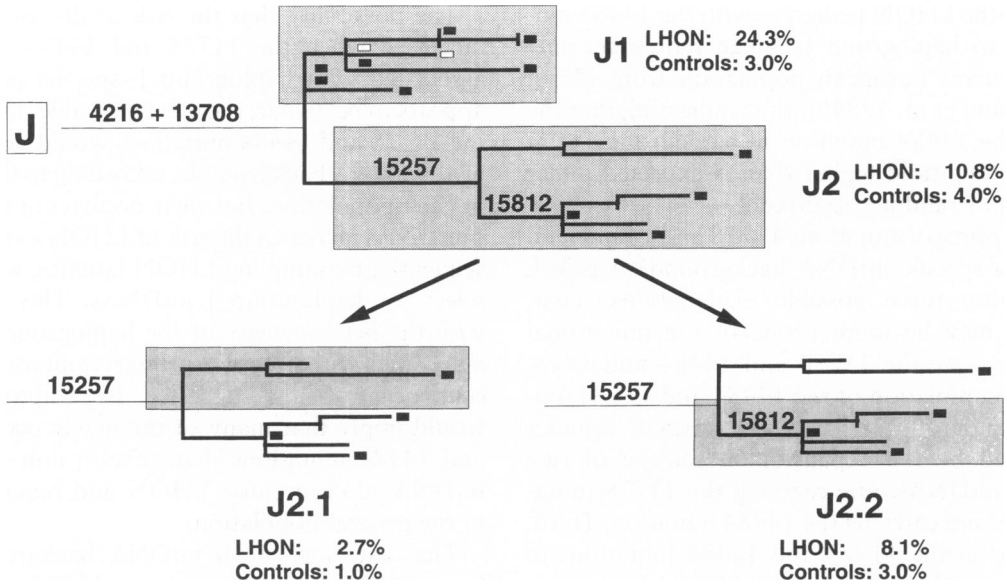

Figure 2 Schematic representation of haplogroup J subclusters and their frequencies in Italian LHON cases and controls. Haplogroup J is divided into the two clusters J_1 and J_2, on the basis of the presence or absence of the 15257 mutation. In turn, the presence or absence of the 15812 mutation subdivides cluster J_2 into the two subclusters $J_{2.1}$ and $J_{2.2}$. Blackened and unblackened rectangles indicate the presence of the 11778 and 14484 mutations, respectively.

results of other studies (Brown et al. 1995; Howell et al. 1995), our data indicate that a large majority of 3460, 11778, and 14484 mutations can be attributed to independent mutational events. The recent occurrence of these mutations is supported further by the high incidence of heteroplasmy that characterizes the Italian LHON families. When we also take into account that the 11778, 3460, and 14484 mutations have been described in Europeans, Africans, and Asians (Wallace et al. 1988; Newman et al. 1994; Ishikawa et al. 1995; Torroni et al. 1996*a*), who harbor a wide range of continent-specific mtDNA backgrounds, these findings further support the primary role played by these mutations in LHON expression. However, phylogenetic analysis also reveals a different distribution of the three primary LHON mutations in the various mtDNA backgrounds.

One extreme is represented by the 3460 mutation, which appears to be distributed randomly among all mtDNA haplogroups. This pattern is expected for a mutation that does not require additional mutations for disease expression and that is eliminated quickly from the population by selection, together with the mtDNAs in which it occurred (Torroni and Wallace 1994). This concept of negative selective pressure for and "quick elimination" of severe LHON mutations is referred to a phylogenetic time scale. These times are very different from those evaluated in the analysis of a few generations of a pedigree (Ghosh et al. 1996) and even from those of related pedigrees sharing a common female ancestor living 12 generations ago (Mackey and Buttery 1992). For instance, the rate of mtDNA evolution of 2.2%–2.9%/million years that was used in this study implies that two identical mtDNAs would require, on average, hundreds of generations in order to acquire a nucleotide difference that is detectable with the restriction enzymes listed in Subjects and Methods. If the haplotype analysis performed in this study were applied to the maternally related pedigrees from Australia, described in the study by Mackey and Buttery (1992), virtually all maternally related subjects would harbor an identical haplotype. Therefore, such a long transmission, in terms of pedigree analysis, would be interpreted as a recent mutational event in phylogenetic analyses.

A different evolutionary pattern is shown by the primary mutation 11778 relative to the primary mutation 3460. The 11778 mutation was observed in a wide range of mtDNA haplogroups but showed an almost sixfold preferential association for the European-specific haplogroup J. Such a preferential association between the 11778 mutation and haplogroup J also is detectable among the European LHON families from North America, described by Brown et al. (1995). An even more extreme pattern is that of the 14484 mutation, which was observed only in association with haplogroup J, in the Italian LHON population. Because of the low frequency of the 14484 mutation in our sample, it was not possible to evaluate the statistical significance of the complete association between the 14484 mutation and haplogroup J. However, recent reanalysis of mtDNA backgrounds described a similar association among Europeans from North America (Brown et al. 1995; Howell et al. 1995; Torroni et al. 1996*a*). In that population,

70%–75% of the LHON pedigrees with the 14484 mutation belong to haplogroup J, which represents only 9% of the general European population from North America (Torroni et al. 1994*b*), thus indicating that the incidence of the 14484 mutation in haplogroup J is at least seven- to eightfold higher than is expected under the hypothesis of random occurrence.

Why do the primary mutations 11778 and 14484 tend to cluster on a specific mtDNA background? A priori, we can envision three possible explanations. First, haplogroup J may be more prone to the mutational events that generate the 11778 and 14484 mutations. Second, the accumulation of the 14484 and 11778 mutations on haplogroup J may reflect ancient founder events followed by the expansion in Europe of two haplogroup J mtDNAs, one carrying the 11778 mutation and the second carrying the 14484 mutation. Third, the occurrence of the 11778 and 14484 mutations in haplogroup J mtDNAs may make LHON expression more likely than in other haplogroups.

The first scenario appears unlikely. Analysis of human mtDNA variation has shown that some specific sequence mutations can increase the mutation rate in adjacent mtDNA regions. For instance, the transition T→C at np 16189 in the control region induces instability of the surrounding cytosines (Horai and Hayasaka 1990). Similarly, an increased length of the stretch of cytosines at nps 568–573 can induce a 270-bp heteroplasmic tandem duplication in the mtDNA control region (Torroni et al. 1994*b*). However, analysis of the mtDNA sequences surrounding nps 11778 and 14484 does not reveal particular features of the surrounding nucleotides that could justify an increased rate of mutation of 11778 and 14484, in haplogroup J. In addition, such a phenomenon perhaps could explain an increased rate of mutation for one nucleotide but not for two nucleotides located in different genes. It also could be argued that the general evolution rate of haplogroup J mtDNAs is increased relative to those of other haplogroups and that all nucleotides, including those at nps 11778 and 14484, are more prone to mutate. However, such an argument is not supported by the observed stability of the marker mutations that define haplogroup J mtDNA in the coding region (+10394 *Dde*I, +4216 *Nla*III, −13708 *Bst*OI, −7474 *Alu*I, −15257 *Acc*I, and −15812 *Rsa*I) and in the control region (16069T, 16126C, and 295T) (Richards et al. 1996; Torroni et al. 1996*b*).

The possibility of ancient founder events also is unlikely, because it would require two founder events both occurring by chance in haplogroup J. In addition, this possibility is not supported either by the heterogeneity of the haplogroup J haplotypes in which the 11778 and 14484 mutations have been observed, in this study and in previous studies (Brown et al. 1995; Howell et al. 1995), or by the presence of heteroplasmy, which suggests recent and multiple mutational events.

The possibility that the risk of disease expression is increased when the 11778 and 14484 mutations are associated with haplogroup J–specific polymorphisms appears to be, by far, the most plausible. In this scenario, the 11778 and 14484 mutations would occur randomly in all mtDNA backgrounds, according to their frequency in each population, but their occurrence in haplogroup J mtDNAs increases the risk of LHON expression. Consequently, by sampling LHON families, we also tend to select for haplogroup J mtDNAs. This is compatible with the heterogeneity of the haplogroup J haplotypes associated with the two primary mutations—their recent origin being suggested by heteroplasmy—and would imply that many of the newly occurring 11778 and 14484 mutations that arise in non–haplogroup J mtDNAs do not cause LHON and remain undetected in the general population.

The detection of an mtDNA background that enhances the penetrance of primary LHON mutations also raises the possibility that specific mtDNA backgrounds may reduce the penetrance of primary mutations. One of these candidate haplogroups may be haplogroup U, a haplogroup that is shared between Europeans and Africans (Torroni et al. 1996*b*) and the presence of which is reduced significantly among the Italian LHON patients with the 11778 mutation. However, additional data from other populations, concerning the relationship between LHON primary mutations and haplogroup U, are necessary for full evaluation of the significance of this observation.

If haplogroup J plays a role in LHON expression by increasing the penetrance of the two primary mutations 11778 and 14484, at least one of the numerous haplogroup J–specific mutations has to be involved in this phenomenon. To shed some light on this issue, we dissected haplogroup J into four subclusters, according to the presence or absence of putative LHON secondary/intermediate mutations (fig. 2). The presence of the 15257 mutation allows the dissection of haplogroup J into two clusters, J_1 and J_2. J_1 lacks the 15257 mutation, which, on the contrary, defines all J_2 haplotypes. The comparison of the frequency ratios of J_1 and J_2 in patients and in controls reveals that the 15257 mutation does not increase at all the risk of LHON expression. Actually, whereas the increased risk for J_1 (characterized by the 4216 + 13708 combination) is ~8-fold, the increased risk for J_2 (characterized by the 4216 + 13708 + 15257 combination) is only 2.7-fold. Moreover, the subdivision of J_2 into two subclusters ($J_{2.1}$ and $J_{2.2}$), according to the presence or absence of the 15812 mutation, reveals that $J_{2.1}$ (characterized by the 4216 + 13708 + 15257 combination) and $J_{2.2}$ (characterized by the 4216 + 13708 + 15257 + 15812 combination) have an unchanged increased risk (2.7-fold) of disease expression. Thus, the 15257 and 15812 mutations do not appear to play any role in increasing the penetrance of the

primary mutations 11778 and 14484, and their increased frequency in 11778- and 14484-positive patients relative to controls simply reflects their association with the 4216 + 13708 combination. This result supports conclusions reported elsewhere that concern the 15257 mutation (Oostra et al. 1994; Mackey et al. 1996) but still leaves open the possibility that the 15257 and 15812 mutations may play a role in disease expression in the limited number of LHON cases lacking the primary mutations 11778, 3460, and 14484. However, our study shows that the 15257 and 15812 mutations are much more common in control populations than was reported in previous studies (Brown et al. 1992*a*). Thus, it also is possible that the LHON cases (Brown et al. 1992*a*; Howell et al. 1993*b*; Johns et al. 1993*b*) in which the 15257 mutation has been observed in the absence of known primary mutations simply reflect the high frequency of this mutation in some Caucasoid populations and that these few cases of LHON are due to some still unknown primary mutations rather than to the 15257 mutation.

The dissection of haplogroup J suggests that the best candidate for increasing the penetrance of primary LHON mutations is the ancient combination 4216 + 13708. Both mutations involve amino acid changes in ND subunits (ND1 and ND5) and could affect the structure, stability, and activity of complex I in the presence of the nucleotide changes caused by the 11778 (ND4) and 14484 (ND6) mutations. However, other ancient missense mutations in the ND genes of haplogroup J also should be reconsidered, including the A→G transition at np 10398 in ND3, which had been dismissed in a previous study (Brown et al. 1992*a*) because of its presence in numerous haplogroups and its high frequency in human populations. Indeed, it is possible that the increased penetrance of primary LHON mutations in haplogroup J is due to the particular combination of multiple amino acid changes in its ND genes rather than to a single amino acid change.

The results of this study point to the following conclusions. First, the preferential association between the primary mutations 11778 and 14484 and haplogroup J, one of the European-specific haplogroups, most likely is due to an effect of this mtDNA background in increasing the penetrance of the two primary mutations. Second, the putative secondary/intermediate LHON mutations 4216, 4917, 13708, 15257, and 15812 are all very common European-specific polymorphisms. Among these mutations, only the 4216 + 13708 combination appears to play a role in LHON expression, being the most likely cause of the increased penetrance of the primary mutations 11778 and 14484 when these occur in haplogroup J mtDNAs. Third, the observation that haplogroup J mtDNAs are more prone to LHON expression suggests that LHON could be more common in Europeans than in Africans and Asians and should reach the highest incidence among European populations with the highest frequency of haplogroup J. Fourth, a differential risk of LHON expression, depending on the mtDNA background in which primary mutations have occurred, also could affect some clinical and biochemical parameters. Until now these parameters usually have been evaluated and compared on the basis of the primary mutation, without the mtDNA background being taken into account. A determination of the effect of mtDNA background on these parameters could be important for our general understanding of the molecular mechanisms of LHON.

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Appendix

Table A1

Haplotypes and Polymorphic Sites Observed in the mtDNA of Italian LHON Patients and Controls

Polymorphic Site(s)[a]	Haplotypes
	LLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLL
	11111111112222222222333333333344444444445555555555566666 11111111112222222222333333333
	12345678901234567890123456789012345678901234567890123456789012345678901234123456789012345678901234567890123456789012345678901234567
54k	00000100000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
259a	00000000000000000000000000000000000010000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
663e	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
717g	00100000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
1154a	11111111111111111111111111111111111011111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
1715c	11111111111111111111111111111111111111111111000000011011111111111111111111111111001111111111111111111
1718a	00000000000000000000000000000000010000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
2208a	11111111111111111111011111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
2293a	00000000000000000000000100000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
2537k	00000000000000000000000000000000000000000000000010000000000000000000000000000000000000000000000000000000000000000000000000000000000
2849k	11111111111111111111111111111111111111111111111101111111111111111111111111111111111111111111111111111111111111111111111111111111111
2856c	11111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111101111111111111111111111111111111111
3337k	11111111111111111111111111111111111111111111111111111111101111111111111111111111111111111111111111111111111111111111111111111111111
3457p	11111111111111111111111111111111111111111111111111111111111111111111111111001111111110001101111111111111101
3534c	11111111111111111111111111111111111111111111111111111101111111111111111111111111111111111111111111111111111111111111111111111111111
3537a	11101111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
3592h	00000000000000000000000000000000000000000000000000000000000000000000000010000000000000000000000000000000000000000000000000000000000
4092e	00000000000000000000000000000000000010000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
4133q	00000000000000000000000000000000000000000000000000000000000000000000000011000000000000000000000000000000000000000000000000000000000
4216q	00000000000011111111000000000000000000000000000000000011111100000000001100000000000111111111101
4529n	11111111111111111111111111111111111111111111111100001111111111111111111111111111111111111111111111111111111111111111111111111111111
4577q	11111111111111111111111111111111111111001111111111111111111111111111111111111101111111111111111111111
4769a	00000000001000000000000000000000000000000000000000000000000000000000000011000000000000000000000000000000000000000000000000000000000
4793e	00000000100000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
4914r	00000000000011111111000000000000000000000000000000000000000000000000000011100000000000000000000000000000000000000000000000000000000
4990a	11111111111111111111111111111111100101111111111111111111111111111111111111111101111111111111111111111
5176a	11111111111111111111111111111111111111111111111111111111111111111111111101111111111111111111111111111111111111111111111111111111111
5198g	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000100000000000
5261e	11111111111111111111111111111111111111111111111111111111111111111111111101111111111111111111111111111111111111111111111111111111111
5584a	11111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111101111111111111111111111111111111111
5978a	11111111111111111111111111111111111111111111111101111111111111111111111111111111111111111111111111111111111111111111111111111111111
6022a	11111111111111101111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
6260e	11111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111101111111111111111111111
7025a	00000000000011111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
7090g	00000000000000000000000000000000000000000000010000000000000000000000000000000000000000000000000000000000000000000000000000000000000
7223c	00000000000000000000000000011100000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
7316j/7319c	00001000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
7474a	11111111111111111111111111111111111111111111111111111111111111111000111111111111111111111111111111111000011111
7852j/7853o	00000010000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
8012k	11111111111111111111111111111111111111111111101111111111111111111111111111111111111111111111111111111111111111111111111111111111111
8090e	00000000000000000000000000000000000000000000000010000000000000000000000000000000000000000000000000000000000000000000000000000000000
8148e	00000000000000000000000000000000000000000000000000000000000000010000000000000000000000000000000000000000000000001000010000
8249b/8250e	00000000000000000000000000000000000011000000001110000000000000000000000000001000010000010000000000
8592j	11111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111101111111111111111111
8616j	11111111111111111111111111111111111111111111111101111111111111111111111111111111111111111111111111111111111111111111
8838e	11111111111111101111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
8858f[b]	11111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
8994e	11111111111111111111111111111111111111001111111111111111111111111111111111111101111111111111111111111
9052n/9053f	11111111111111111111111111111111111111111111111110001111111111111111111111111110001111111111111
9156e	00010000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
9246e	00000000000000000001000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
9272c	11111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
9714e	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
10028a	00000000000000000000000000000000000000000000000011110000000000000000000000000000000000000000000000000000000000000000000000000000000
10289a	00000000000000000000000000000000000010000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
10352e	00000000000000000000000000010000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
10364e	11111111111111111111111111111101111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
10394c	00000000000000000000000000000000000000000000000011110100111111110000000000000000000011111111111111101
10397a	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
10806g	00000000000000000000000000000000000000000000000000000000000000000000000011000000000000000000000000000000000000000000000000000000000
11001n/11002f	00000000000000000000000000000000000000000000000000000000000000010000000000000000000000000000000000000000000000000100000000
11362a	11111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111101111111111111111111

(*continued*)

Table A1 (continued)

Polymorphic Site(s)[a]	Haplotypes
	LLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLL
	1111111111222222222233333333334444444444555555555566666 1111111111222222222233333333
	1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567
11421l	11111111111111111111111111111111111101111111111111111111111111111111111111111111111111111111111111111
11439j	00000000000000000010000000000000000000000000000000000000000000000000000000000000000000000000000000000
11775s	00000000000000000000000000000000000000000000000000000000000000000000011001111111100011011111111111110001
12026o/12026h	00000000000000000000000000000000000000010000000000000000000000000000000000000000000000000000000000000
12308g	00000000000000000000111111111111111100000000000000011110000000000000000000110000011100000000000010
12629b	11111111111111110111111111111111111111111111111111111111111111111111111111111011111111111111111111111
12810k	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
12898g	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000001000000
12925j	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000100000
13031g	11111111111111111111111111111111111111111111101111111111111111111111111111111111111111111111111111111
13103g	11111111111111111111111111111111101111111111111111111111111111111111111111111111111111111111111111111
13103g/13104j	00000000000000000000000000000001001000000000000000000000000000000000000000000000000000000000000000000
13366m/13367b/13367j	00000000000111111100000000000000000000000000000000000000000000000000000000000000000000000000000000000
13635l	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
13702e	00000000001000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
13704t	11111111111111111101111111111111111111111111111111111111111111111111111111111111111111111111111111111
13730g	00000000000000000000000000000000001000000000000000000000000000000000000000000000000000000000000000000
13803e	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
14015a	11111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
14068l	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
14139j	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
14199o[b]	11111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
14258m	11111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
14268g[b]	11111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
14276t/14279e	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
14368g[b]	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
14465u	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
14484t	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
14724k	00000000000000000000000001000000000000000000000000000000000000000000000000000000000000000000000000000
15254u	11111111111110111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
15606a	00000000000011111111000000000000000000000000000000000000000000000000000000000000000000000000000000000
15614n/15615f	00000000010000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
15812k	11111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
15872k	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
15925i	11111111111100000000111111111111111111111111111111111111111111111111111111111111111111111111111111111
16049k	11111111111111111111111111111111011111111111111111111111111111111111111111111111111111111111111111111
16065g	11111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
16143l	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
16145j	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
16170j	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
16208k	11111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
16254a	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
16303k	11111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
16310k	11111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
16389m/16390b/16390j	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
16389g/16390b	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
16398e	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
16517e	10101111110111111111000101001010000101100010011111111111100001011111111111100100111111010101101000001
12S rRNA insertion	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
9-bp deletion	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000
9-bp triplication	00000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000

Note.—One hundred seven polymorphic sites, the 9-bp COII/tRNA^{Lys} intergenic deletion, the 9-bp triplication in the same region, and the 12S rRNA gene insertion of five cytosines in the region between nps 956 and 965 define the mtDNAs observed in 37 Italian LHON patients (L1–L37) and the 64 mtDNA haplotypes (1–64) observed in 99 Italian controls (51 continental Italians and 48 Sardinians). A "1" indicates the presence of a site and a "0" indicates the absence of a site, except for the 12S rRNA gene insertion, the 9-bp COII/tRNA^{Lys} deletion, and the 9-bp COII/tRNA^{Lys} triplication, in which a "1" indicates the presence of the insertion, the deletion, or the triplication, respectively.

[a] Numbered from the first nucleotide of the recognition sequence, according to the published sequence (Anderson et al. 1981). The restriction enzymes used in the analysis are designated by the following single-letter codes: a = *Alu*I; b = *Ava*II; c = *Dde*I; e = *Hae*III; f = *Hba*I; g = *Hin*fI; h = *Hpa*I; i = *Hpa*II; j = *Mbo*I; k = *Rsa*I; l = *Taq*I; m = *Bam*HI; n = *Hae*II; o = *Hin*cII; p = *Bsa*HI; q = *Nla*III; r = *Bfa*I; s = *Mae*III; t = *Bst*OI; and u = *Acc*I. Sites separated by a slash (/) indicate either simultaneous site gains or site losses for two different enzymes or a site gain for one enzyme and a site loss for another because of a single inferred nucleotide substitution; these sites are considered to be only one restriction-site polymorphism in the parsimony analysis. Underlined sites indicate mutations that have been associated with LHON.

[b] Found to be present or absent in all samples, contrary to the published sequence (Anderson et al. 1981).

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